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The C-Terminal Domain of Cernunnos/XLF Is Dispensable for DNA Repair In Vivo▽†

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The core nonhomologous end-joining DNA repair pathway is composed of seven factors: Ku70, Ku80, DNA-PKcs, Artemis, XRCC4 (X4), DNA ligase IV (L4), and Cernunnos/XLF (Cernunnos). Although Cernunnos and X4 are structurally related and participate in the same complex together with L4, they have distinct functions during DNA repair. L4 relies on X4 but not on Cernunnos for its stability, and L4 is required for optimal interaction of Cernunnos with X4. We demonstrate here, using in vitro-generated Cernunnos mutants and a series of functional assays in vivo, that the C-terminal region of Cernunnos is dispensable for its activity during DNA repair.

Nonhomologous end joining (NHEJ) represents the main pathway for solving DNA double-strand breaks (DSB) in mammals. The core of the NHEJ pathway is composed of seven proteins: Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis, XRCC4 (X4), DNA ligase IV (L4), and Cernunnos/XLF (Cernunnos) (reviewed in reference 18). Briefly, the Ku70-Ku80 heterodimer bound to broken DNA recruits the serine/threonine kinase DNA-PKcs. DNA-PK phosphorylates downstream effectors such as the nuclease Artemis. The X4-L4 complex carries out the final joining of synapsed DNA ends in association with Cernunnos (2, 6). Cernunnos was identified through cDNA functional complementation of a fibroblast cell line obtained from a human patient with immune deficiency and microcephaly (5). The same factor, called XLF, was identified through a yeast two-hybrid screen with X4 as a bait (2).

Cernunnos is structurally related to X4 and consists of a globular head domain followed by a coiled-coil region and an unstructured C-terminal domain (2, 6, 12). One major difference between the structures of X4 and Cernunnos appears in the coiled-coil region. While this region is linear in X4, a hinge in the middle of the coiled-coil of Cernunnos folds back the end of the domain toward the head (3, 14).

Cernunnos interacts with the X4-L4 complex in vivo and in vitro (2, 6). Cernunnos and X4 both appear to interact directly with L4, but the Cernunnos-L4 interaction seems to be very weak (7). In addition, purified Cernunnos associates with DNA in a sequence-independent manner (20) but in a DNA length-dependent manner, like X4 (15). Although the X4-L4 complex can ligate DNA in vitro (10), Cernunnos further improves this activity (11, 15, 16, 20). Cernunnos seems important, in particular, for the ligation of mismatched or noncohesive DNA ends, but not for that of compatible DNA ends, in vitro (10, 20).

Cernunnos is therefore a "core" NHEJ component, but limited information is available about its precise function during DNA repair in vivo. We show here that although X4 and Cernunnos share sequence and structural homologies, their functions are distinct. We also demonstrate that Cernunnos requires L4 for its association with X4. Lastly, the Cernunnos C terminus is dispensable for DNA repair following ionizing radiation (IR) and V(D)J recombination.

MATERIALS AND METHODS

Cells. Cernunnos-deficient cells (from patient P2 in reference 5) and OTel control cells, which are skin fibroblasts (17), are transformed with simian virus 40 and immortalized with telomerase. X4 knockout (KO) and wild-type (WT) mouse embryonic fibroblasts (MEFs), generated from embryos at embryonic day 14.5, were immortalized after cell transfection with plasmid pLasWT expressing simian virus 40 T antigen as described previously (23). The L4-defective N114P2 cells and the parental cell line Nalm6 were gifts from M. R. Lieber (9).

Cloning. WT Cernunnos, deletion mutants (see Fig. 3A), and the WT X4 Orf were PCR amplified from a cDNA library and cloned into the pcDNA3.1 vector (Invitrogen). Chimeras C1X2, X1C2 (see Fig. 4A), and C1GFP (see Fig. S7A in the supplemental material) were obtained using a double PCR amplification procedure and were cloned into the same vector. All constructs were subcloned into the pMND-Myc-internal ribosome entry site (IRES)-green fluorescent protein (GFP) retroviral vector by using the BD In-Fusion protocol (Clontech).

V(D)J recombination assays. In-chromosome V(D)J recombination assays were performed as previously described (5, 17). For complementation analysis, 2.5 μg of the plasmid of interest was cotransfected during the V(D)J assay. Extrachromosomal V(D)J recombination assays were performed with pBlueRec (13) in MEFs or with the pRecCS substrates (5) in human fibroblasts.

WB and IP experiments. Cernunnos, X4, truncation mutants, and chimera-expressing constructs were used to transfect 293T cells. Immunoprecipitations (IP) were performed as described previously (6) on precleared lysates using polyclonal rabbit anti-immunoglobulin G (anti-IgG) (Santa Cruz), anti-V5 tag (Abcam), anti-Myc tag (Sigma-Aldrich), anti-L4 (also used for revelation; Abcam), and anti-X4 (Serotec) antibodies. Immunoprecipitates were analyzed

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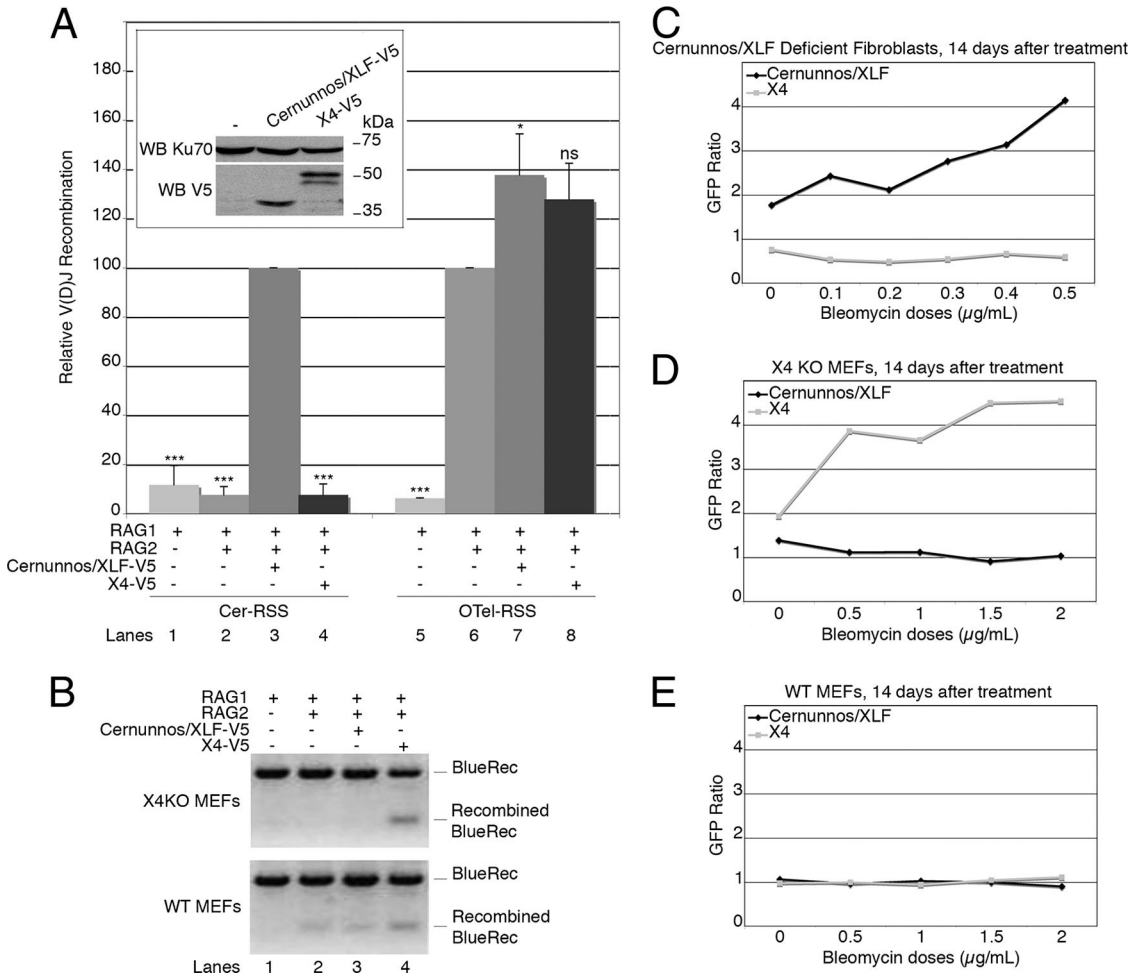

FIG. 1. Evidence for distinct functions of Cernunnos and X4. (A) Mean results of three V(D)J recombination assays on chromosomal substrates integrated into Cernunnos-deficient fibroblasts (Cer-RSS) and WT OTel fibroblasts (OTel-RSS). Relative V(D)J recombination is calculated based on the recombination frequency obtained by transfection with RAG1 and RAG2 for OTel-RSS and with RAG1, RAG2, and Cernunnos for Cer-RSS cells. ns, nonsignificant difference ($P > 0.005$); *, statistically significant difference ($P < 0.005$); ***, highly statistically significant difference ($P < 0.001$). (Inset) Expression of V5-tagged Cernunnos and X4 constructs in 293T cells. (B) V(D)J recombination assay in X4KO and WT MEFs using the pBlueRec system. Recombination of the substrate was analyzed by PCR. (C, D, and E) Survival of Cernunnos-deficient cells (C) and X4KO (D) and WT (E) MEFs transduced with the pMND retrovirus containing Cernunnos or X4 14 days after bleomycin treatment.

by Western blotting (WB) with murine antibodies directed against the V5 tag (Invitrogen), the Myc tag (Santa Cruz), and X4 (Genetex).

Bleomycin sensitivity assay. Virus supernatants, produced from pMND-Myc-IRES-GFP constructs, containing Cernunnos (WT or 1-230, 1-178, 1-167, or 1-141 mutant [where numbers represent the positions of the amino acids in each mutant]) cDNA or WT or chimeric (C1X2 or X1C2) X4 cDNA, were used to transduce Cernunnos-deficient cells or X4KO or WT MEFs as described previously (5) in order to generate stable cell lines expressing C-terminal Myc-tagged proteins and GFP. Established cell lines were incubated at 37°C with medium alone or with medium containing increasing doses of bleomycin for 1 h, after which the medium was replaced by complete medium. Cells were split regularly and analyzed for GFP expression by fluorescence-activated cell sorting over 14 days (Becton Dickinson FACScan flow cytometer). The ratio of GFP^- (untransduced) to GFP^+ (transduced) cells at the initiation of culture was set to 1.

Immunofluorescence detection of IR-induced foci (IRIFs). Cernunnos-deficient fibroblasts were transduced with the empty pMND-Myc-IRES-GFP retroviral vector or the pMND-Myc-IRES-GFP vector containing Cernunnos (WT or the 1-230 deletion mutant), seeded on cover slides, and gamma-irradiated (2 Gy). DNA repair foci were labeled either with a monoclonal mouse anti-γH2AX antibody or a polyclonal rabbit anti-53BP1 antibody, revealed by an Alexa Fluor 546-coupled goat anti-mouse or anti-rabbit secondary antibody (Molecular Probes), 2 h and 24 h after IR, as described previously (5). Slides were counterstained with 0.1 μg/ml of DAPI (4′,6′-diamidino-2-phenylindole), mounted in Fluorsave (Calbiochem), and viewed by epifluorescence microscopy.

RESULTS AND DISCUSSION

Separate functions of Cernunnos and X4. We analyzed the respective roles of X4 and Cernunnos during V(D)J recombination by using an in-chromosome V(D)J recombination assay (5). The Cernunnos-deficient cell line carrying an intrachromosomal substrate (Cer-RSS) rearranges the substrate upon transfection with RAG1 and RAG2 and ectopic expression of WT Cernunnos (set as 100% efficiency), as expected, but not upon overexpression of X4 (Fig. 1A). Conversely, X4 but not Cernunnos complements the V(D)J defect in X4KO MEFs in a V(D)J extrachromosomal assay (Fig. 1B). During general DNA repair, a selective advantage is conferred by ectopic expression of Cernunnos only in Cernunnos-deficient human

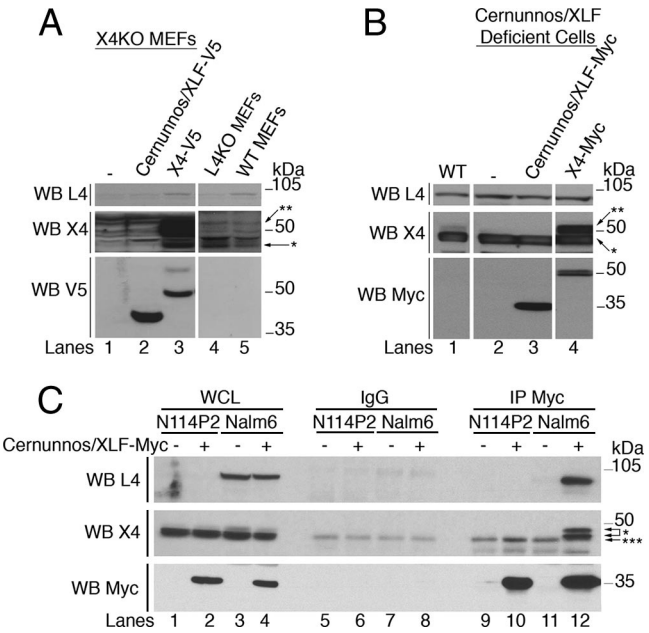

FIG. 2. The Cernunnos-X4-L4 complex. (A) Whole-cell lysates (WCL) from X4KO MEFs, either left untransfected or transfected with a V5-tagged Cernunnos or X4 expression construct, and from L4KO and WT MEFs were analyzed by WB with the indicated antibodies. (B) WCL from WT fibroblasts and Cernunnos-deficient cells transfected with a V5-tagged Cernunnos or X4 expression construct as for panel A. (C) WCL from N114P2 (L4KO) or Nalm6 (parental) cells, transduced (+) or not (−) with pMND-Cernunnos-Myc-IRES-GFP, were immunoprecipitated with irrelevant antibodies (IgG) or anti-Myc antibodies and then blotted and revealed as indicated. *, endogenous X4; **, V5-tagged X4; ***, IgG heavy chain.

fibroblasts and by ectopic expression of X4 only in X4KO MEFs upon treatment with the radiomimetic drug bleomycin, as manifested by the dose-response increase in the GFP ratio following treatment (Fig. 1C and D).

We conclude that Cernunnos and X4, although structurally related, exert specific and not interchangeable functions during DNA DSB repair. This is in accord with the lack of redundancy of these two factors in vivo, both in KO mice and in humans. Attesting to their specific functions during DNA repair, their crystal structures reveal important differences (3, 14). Notably, X4 has a linear coiled-coil domain with which L4 interacts, whereas the Cernunnos coiled-coil forms a hinge that folds back the C terminus of the coiled-coil toward the head domain. The functional consequence of this major difference is not clearly established yet, but at least, it would mask the sequence known in X4 to correspond to the L4-interacting region.

Interactions within the ligation complex. Cernunnos, X4, and L4 are part of the same complex (2, 6, 21). It is also known that in the absence of X4, L4 is not stable (4). A defect in Cernunnos protein does not result in L4 destabilization (6) (Fig. 2B), and the overexpression of Cernunnos in X4KO MEFs could not rescue L4 stability (Fig. 2A). The ectopic expression of Cernunnos or X4 has no effect on L4 expression in Cernunnos-deficient cells (Fig. 2B). We conclude that L4 does not rely on Cernunnos for its stability in vivo. This is in accord with a weak Cernunnos-L4 interaction (3, 15), even though surface plasmon resonance studies using purified proteins in vitro have demonstrated such an interaction (14).

To further understand their interdependence within the X4-L4-Cernunnos complex, we analyzed the behavior of X4 and Cernunnos in the absence of L4 by using the N114P2 cell line, an L4-defective derivative of the human Nalm6 pre-B-cell tumor (9). IP of exogenous Cernunnos protein from WT Nalm6 cells coprecipitated both X4 and L4 (Fig. 2C), as previously described (2, 6). In contrast, IP of Cernunnos from the L4-deficient N114P2 cells failed to pull down endogenous X4 to a detectable level. Knockdown of L4 by short hairpin RNA has been shown to reduce the recruitment of Cernunnos to the site of DNA damage (21). The opposite is not true, since both X4 and L4 are recruited at DNA DSB in the absence of Cernunnos.

We conclude from these experiments that the formation of a complete X4-L4-Cernunnos complex relies both on X4 for the stabilization of L4 and on L4 for the inclusion of Cernunnos within the complex. Given the degradation of L4 in X4-deficient cells (4), it is not possible to analyze directly the relation of L4 and Cernunnos in the absence of X4.

Important domains of Cernunnos. Given the evolutionary relation between X4 and Cernunnos and their specific functions during DNA repair, we aimed at identifying their various functional domains by generating Cernunnos deletion mutants and X4-Cernunnos chimeras through domain swapping. In all cases, the resulting mutants were analyzed for their stability, their participation in the formation of X4-L4-Cernunnos complexes, their activity during V(D)J recombination, and their capacity to ensure general DNA DSB repair in vivo.

We previously divided the Cernunnos protein into three structurally distinct domains based on analysis of its sequence (6): the head (amino acids [aa] 1 to 141), a coiled-coil central region (aa 142 to 230), and a nonstructured C-terminal region (aa 231 to 299) (Fig. 3A). Only the full-length and C-terminus-deleted forms of Cernunnos complemented the V(D)J defect in Cer-RSS cells (Fig. 3B) in transient transfection assays; all the other deletion mutants, extending within the coiled-coil central region, demonstrated sharp decreases in their activities. This was particularly the case for the 1-178 and 1-167 mutants, which recapitulate mutations identified in Cernunnos patients (5). Likewise, the Cernunnos head-only construct (1-141) was inefficient in V(D)J recombination, but its mirror form (the 142-299 construct) could not be tested due to poor stability (data not shown).

To differentiate between the qualitative and quantitative consequences of these various mutations, we performed a pilot experiment to determine the minimal amount of WT Cernunnos protein required to fully complement Cer-RSS cells for V(D)J recombination and detection of the protein by WB in 293T cells (see Fig. S1 in the supplemental material). Several situations can be noted: full complementation is obtained by overexpression (4 μg to 2.5 μg of plasmid), and the level of complementation then decreases slowly to 60% efficiency when the amount of plasmid is decreased to 1 μg. By use of an amount of plasmid with which the protein is no longer detectable by WB (1 μg to 0.05 μg), the activity remains constant at 60%. Lastly, below 0.01 μg of plasmid, the V(D)J activity is no longer complemented. In summary, one can still achieve statistically significant complementation of the V(D)J recombi-

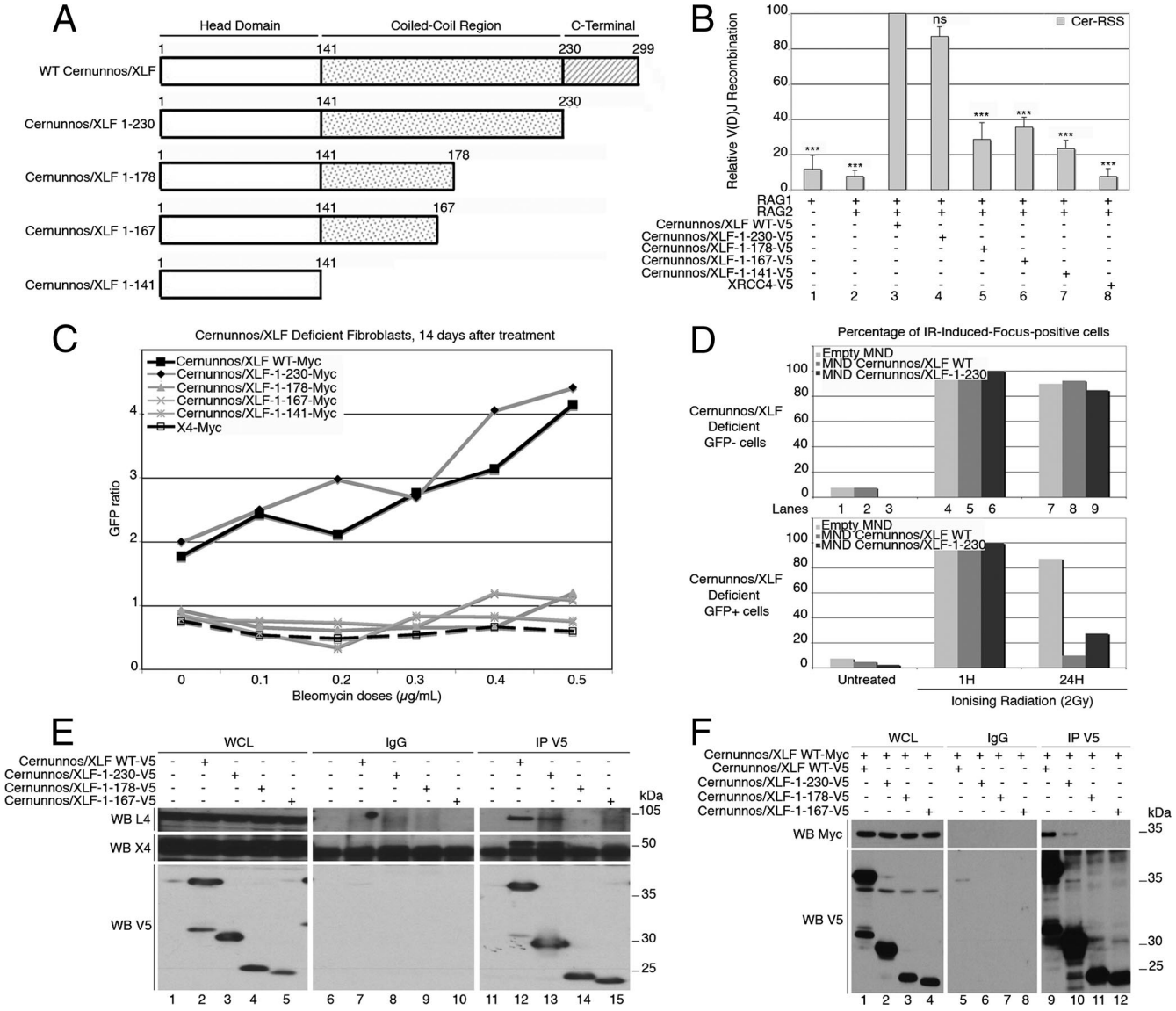

FIG. 3. Analysis of Cernunnos domains. (A) Schematic representation of WT Cernunnos and its mutants. (B) Mean results of three V(D)J recombination assays on chromosomal substrates integrated into Cernunnos-deficient cells (Cer-RSS), calculated as described in the legend to Fig. 1A. (C) Survival of Cernunnos-deficient cells, transduced with the pMND vector containing WT or mutant (1-230, 1-178, 1-167, or 1-141) Cernunnos or WT X4, 14 days after bleomycin treatment. Results are expressed as in Fig. 1C. (D) Percentage of IRIF-positive cells. Forty cells, transduced (GFP^+) or not (GFP^-) and either left untreated or subjected to IR, were scored for DAPI, GFP, and γH2AX foci 2 h and 24 h after IR (2 Gy). A cell was considered IRIF positive if it had more than 10 IRIFs. (E) Whole-cell lysates (WCL) from 293T cells transfected with V5-tagged WT or mutant (1-230, 1-178, or 1-167) Cernunnos were immunoprecipitated with irrelevant (IgG) and anti-V5 rabbit antibodies. (F) 293T cells were cotransfected with Myc-tagged WT Cernunnos and V5-tagged WT or mutant (1-230, 1-178, or 1-167) Cernunnos. For panels E and F, the immunoprecipitates were analyzed by WB as indicated.

nation in Cer-RSS cells with an amount of Cernunnos plasmid that does not lead to detectable expression of the protein in 293T cells. Since the deletion mutants for which test results are shown in Fig. 3 are detected comparably to the WT form in 293T cells, their loss of activity is most likely qualitative rather than due to the modulation of their expression level.

To gain further insight into the functionality of these truncation mutants, we analyzed their propensity to confer a selective growth advantage on Cernunnos-deficient cells upon treatment with increasing doses of bleomycin (Fig. 3C). A selective advantage, as judged by an increase in the frequency of GFP^+ transduced cells, was obtained with the WT and the 1-230 construct, while the GFP ratio did not change with the other mutants. Lastly, transduction of Cernunnos-deficient cells with Cernunnos-IRES-GFP resulted in the disappearance of γH2AX IRIFs (a hallmark of NHEJ deficiency [5]) in GFP^+ transduced cells 24 h after IR (Fig. 3D; see also Fig. S2 in the supplemental material), attesting to efficient DNA repair in these cells. Likewise, the efficient DNA repair obtained with the Cernunnos 1-230 mutant was attested to by the disappearance of γH2AX foci 24 h following IR.

We determined the level of Cernunnos expression upon

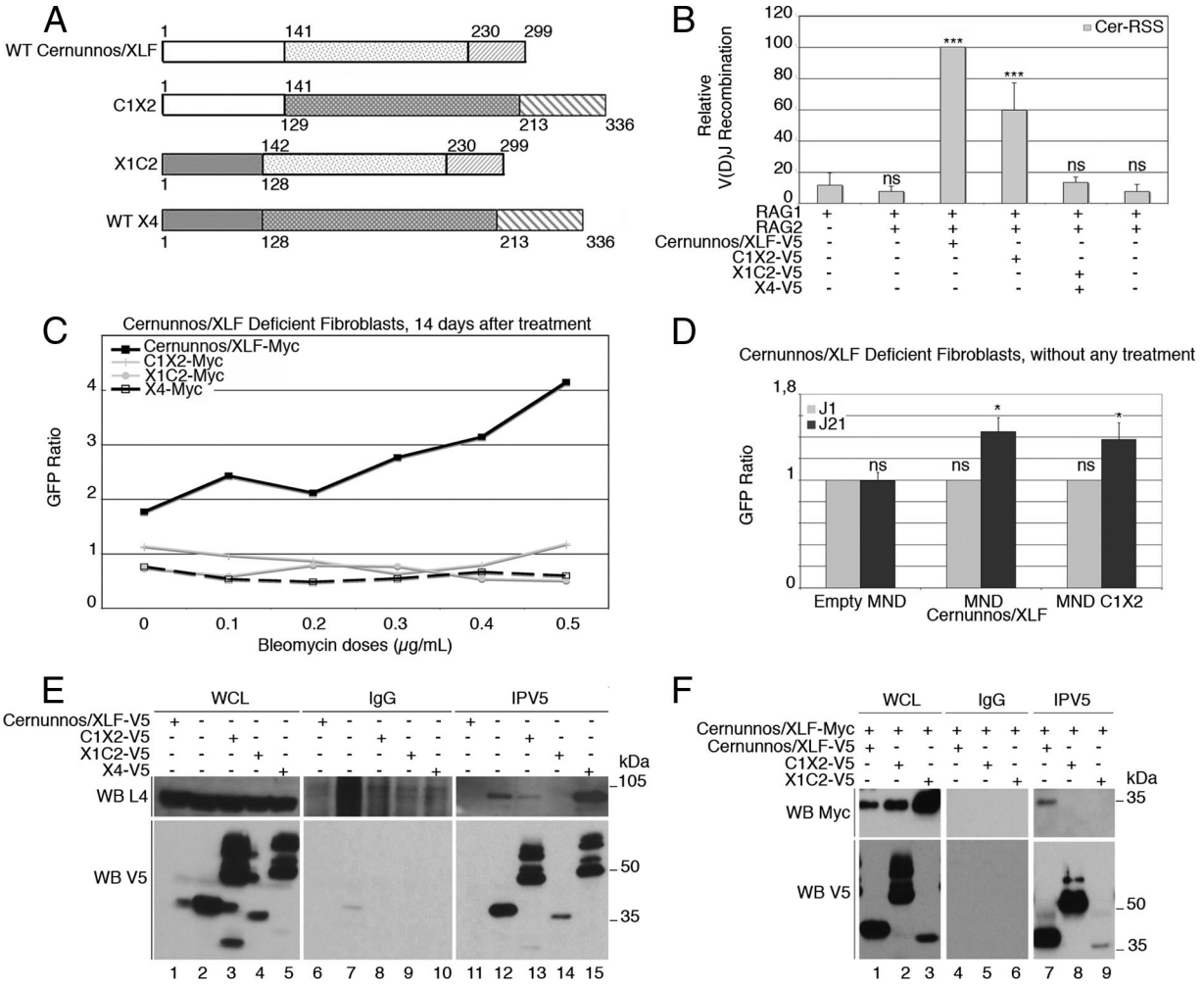

FIG. 4. Analysis of chimeras between Cernunnos and X4. (A) Summary of chimeras resulting from swapping of domains between WT Cernunnos and X4. (B) Mean results of three V(D)J recombination assays on chromosomal substrates integrated into Cernunnos-deficient cells (Cer-RSS). Results are expressed as described in the legend to Fig. 1A. (C) Survival of Cernunnos-deficient cells, transduced with the pMND-Myc-IRES-GFP vector containing WT Cernunnos, C1X2, X1C2, or WT X4, 14 days after bleomycin treatment. Results are expressed as in Fig. 1C. (D) Survival of Cernunnos-deficient cells transduced with an empty MND vector or with the vector carrying WT Cernunnos or C1X2, without any treatment. Cells were monitored for 21 days. J1, day 1; J21, day 21. Asterisks indicate statistical significance ($P < 0.05$). (E) 293T cells were transfected with V5-tagged WT Cernunnos, C1X2, X1C2, or WT X4. Whole-cell lysates (WCL) were immunoprecipitated with irrelevant (IgG) and anti-V5 rabbit antibodies. (F) 293T cells were cotransfected with Myc-tagged WT Cernunnos and V5-tagged WT Cernunnos, C1X2, or X1C2. For panels E and F, WCL were treated as for Fig. 3E.

retroviral transduction in fibroblasts by performing quantitative WB analysis (see Fig. S3 in the supplemental material). After normalization to the level of endogenous Ku70 expression, it appears that the exogenous Cernunnos-Myc level in transduced Cernunnos-deficient cells is only about 1.17-fold higher than the endogenous Cernunnos level in WT cells. Complementation by the Cernunnos 1-230 construct is therefore not caused by strong overexpression.

Altogether, these experiments establish that the C-terminal region (aa 231 to 299) of Cernunnos is dispensable for its full DNA repair activity in vivo. In contrast to that of the 1-230 mutant, the activities of the other truncated forms could not really be tested (Fig. 3B, C, and D) due to protein instability (Fig. 3E and F). Although they were expressed to detectable levels in 293T cells, they were undetectable by either WB or immunohistochemistry (see Fig. S4A and B in the supplemental material) in Cernunnos-deficient cells. This could explain the lack of complementation in these cells. IP studies (Fig. 3E and F) demonstrated that the Cernunnos 1-230 mutant can integrate the X4-L4 complex (Fig. 3E) and is capable of heterodimerization with full-length Cernunnos (Fig. 3F). This is in accord with the results of interaction studies using purified proteins in vitro (3) but contrasts with those of studies with *Saccharomyces cerevisiae*, which suggested that the C terminus of Nej1, the yeast ortholog of Cernunnos (6, 8), is required for interaction with Lif1p, the X4 ortholog (19).

The unstructured 68-aa C-terminal region confers DNA binding activity on Cernunnos in vitro (3). Moreover, this region is the site of the DNA-PK- and ATM-dependent phosphorylation of several serine residues (S245 and S251 being the major phosphorylation sites) (22). Yet alanine substitution at these phosphorylation sites (22) or the deletion of the entire

region (our study) has no detrimental impact on NHEJ in vivo, suggesting that this in vitro DNA binding activity may not be relevant during NHEJ. Indeed, the X4-L4 complex is even targeted at DNA breaks in the absence of Cernunnos (21). This strikingly contrasts with NHEJ in yeast, where it was shown that deletion of the C-terminal region of Nej1 severely compromised NHEJ activity (1). Lastly, the C-terminal region of Cernunnos does not participate in the overall three-dimensional structure of the protein, as evidenced by the fact that crystals were obtained with C-terminus-deleted proteins (3, 14). The role of the C-terminal region in other putative functions of Cernunnos will await further studies.

Swapping between Cernunnos and XRCC4. We performed domain-swapping experiments to define the regions of X4 and Cernunnos critical for their functions in DNA repair. In the C1X2 chimera (Fig. 4A), we associated the head (aa 1 to 141) of Cernunnos with the coiled-coil and C-terminal domain of X4, while in X1C2, the head of Cernunnos was replaced with that of X4. The C1X2 chimera complemented the V(D)J recombination defect of Cer-RSS cells to about 60% of the WT level (Fig. 4B), whereas the X1C2 chimera was inefficient. However, none of the chimeras were proficient at complementing the DNA repair defect following bleomycin treatment, as judged by the lack of any increase in the GFP index in mixed-population studies (Fig. 4C). This suggests that the "head" of Cernunnos hooked to the "body" of X4 has enough activity to deal with a few DNA lesions [V(D)J recombination] but is not active enough to manage the many bleomycin-induced DNA DSB.

Since the Cernunnos head is not stable, we verified that the activity of the C1X2 chimera was not solely the consequence of protein stabilization by the X4 coiled-coil by replacing this region with that of GFP (C1GFP) (see Fig. S5 in the supplemental material). The C1GFP protein failed to complement either V(D)J recombination activity or DNA repair (tested by the persistence of IRIFs); this failure was not due to a mislocalization or destabilization of this protein.

Accordingly, C1X2 conferred a slight spontaneous selective advantage on Cernunnos-deficient cells (in the absence of an exogenous DNA DSB inducer), comparable to that obtained with WT Cernunnos (Fig. 4D). The absence of X1C2 activity in Cernunnos-deficient cells could be caused either by the very low stability of X1C2 compared to those of C1X2 and WT Cernunnos (Fig. 4E and F) or by its lack of an important region present in the X4 coiled-coil domains (such as the L4 interaction region) or the head of Cernunnos. Surprisingly, while the C1X2 chimera could associate with endogenous L4 to some extent (Fig. 4E), it did not heterodimerize with WT Cernunnos (Fig. 4F), arguing for a role of the coiled-coil region in Cernunnos dimerization. This lack of heterodimerization could participate in the low functional activity of this chimera. While the C1X2 chimera was partly functional in Cernunnos-deficient cells, it was completely inefficient in complementing the absence of X4 in MEFs from X4KO mice. C1X2 also failed to complement V(D)J recombination activity in X4KO MEFs (see Fig. S6 in the supplemental material) and to confer a selective advantage following bleomycin treatment (see Fig. S7 in the supplemental material), in contrast to the phenotype reversion obtained with WT X4 in both cases.

Altogether, these findings demonstrate that the heads and coiled-coil regions of Cernunnos and X4 are not interchangeable, and they suggest specific roles for each in NHEJ. While a particular function of the coiled-coil region of X4 has been deciphered in the binding of L4, the precise functions of the heads have yet to be determined, possibly through the identification of additional partners.

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